MOLECULAR AND CELLULAR BIOLOGY, Jan. 1987, p. 1–6
0270-7306/87/010001-06$02.00/0

Vol. 7, No. 1

Autonomous Replicating Sequences from Mouse Cells Which Can Replicate in Mouse Cells In Vivo and In Vitro

HIROYOSHI ARIGA,* TERU ITANI, AND SANAE M. M. IGUCHI-ARIGA

Institute of Medical Science, The University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108, Japan

Received 16 July 1986/Accepted 25 September 1986

We have already reported that the cloned mouse DNA fragment (pMU65) could replicate in a simian virus 40 T antigen-dependent system in vivo and in vitro (H. Ariga, Z. Tsuchihashi, M. Naruto, and M. Yamada, Mol. Cell. Biol. 5:563–568, 1985). The plasmid p65-tk, containing the thymidine kinase (*tk*) gene of herpes simplex virus and the *Bgl*II-*Eco*RI region of pMU65 homologous to the simian virus 40 origin of DNA replication, was constructed. The p65-tk persisted episomally in tk^+ transformants after the transfection of p65-tk into mouse FM3A^{tk-} cells. The copy numbers of p65-tk in FM3A^{tk+} cells were 100 to 200 copies per cell. Furthermore, the p65-tk replicated semiconservatively, and the initiation of DNA replication started from the mouse DNA sequences when the replicating activity of p65-tk was tested in the in vitro DNA replication system developed from the FM3A cells. These results show that a 2.5-kilobase fragment of mouse DNA contains the autonomously replicating sequences.

The replication of eucaryotic DNA occurs by initiation at many chromosomal sites which are usually clustered in a specific region characteristic of the cell type and the stage of the cell cycle (18). Recently, several investigators have tried to isolate replication origins by using in vitro recombination techniques (6). Another approach to the isolation of replication origin is to search autonomous replicating sequences (ARSs). Many ARSs of *Saccharomyces cerevisiae*, as well as those of other eucaryotic organisms, have been described (7, 9, 13–15, 17, 20, 22–24). These sequences from higher organisms may act as initiators of replication in *S. cerevisiae* cells. However, there is no evidence that these cloned eucaryotic DNA segments can also act as replicators in the cells of their original higher organisms. We have already reported that the cloned mouse DNA fragment pMU65 can replicate in a simian virus 40 (SV40) T antigen-dependent system in vivo and in vitro (5). The 2.5-kilobase (kb) mouse DNA fragment of pMU65 hybridized to half of a 27-nucleotide perfect palindrome present in the origin of SV40 DNA replication, and the initiation of DNA replication started from this fragment in an SV40 DNA replication system in vitro (5).

We constructed the chimeric plasmid (p65-tk) containing 2.5 kb of mouse DNA, the thymidine kinase (*tk*) gene of herpes simplex virus type 1, and plasmid pKB111. The p65-tk plasmid persisted episomally in transfected mouse FM3A cells and had a good template activity in an in vitro DNA replication system that we have developed from FM3A cells.

MATERIALS AND METHODS

Plasmid and recombinant DNA constructions. Plasmid pMU65 harboring the mouse genomic DNA was reported previously (5) and is shown in Fig. 1. The 2.5-kb *Eco*RI-*Bgl*II fragment of pMU65 was inserted into the *Eco*RI-*Bgl*II region of pKSV10, and the *tk* gene of herpes simplex virus type 1 (10) was further inserted into the *Bam*HI site of the above plasmid. This was named p65-tk (Fig. 1).

Replication of plasmid DNA in FM3A cells. To check the replication of plasmid DNA, mouse FM3A^{tk-} cells (1) were transfected with p65-tk by a liposome-mediated gene transfer technique. This novel technique will be reported elsewhere (T. Itani, T. Yasuda, T. Tadakuma, and H. Ariga, submitted for publication). Briefly, 10^6 FM3A^{tk-} cells grown in Dulbecco modified Eagle medium containing 10% fetal calf serum were transfected with 10 μg of p65-tk in a liposome composed of 1 μmol of phosphatidylserine and seeded at 10^3 cells per well of 24-well plate. After 18 h, the medium was replaced by HAT medium. The HAT medium was changed every 3 days. After 2 weeks, the tk^+ colonies were picked up and cultured in the same HAT medium. The approximately 10^6 cells of each colony were harvested, and the low-molecular-weight DNAs were extracted by the Hirt procedure (12). The DNA in the Hirt supernatant was deproteinized with 100 μg of proteinase K at 37°C for 90 min, fractionated by 0.8% agarose gel electrophoresis, transferred to a nitrocellulose filter (21), and hybridized to a ^{32}P-labeled p65-tk probe. The hybridization was carried out in a mixture containing 3× SSC (1× SSC contains 1.5 M NaCl and 0.15 M sodium citrate), 50 mM Tris (pH 7.5), 1 mM EDTA, 1× Denhardt solution (1× Denhardt solution contains 0.02% Ficoll, 0.02% polyvinylpyrolidone, and 0.02% bovine serum albumin), 20 μg of tRNA per ml, 20 μg of heat-denatured salmon testis DNA per ml, ^{32}P-labeled p65-tk, 0.1% sodium dodecyl sulfate, and 50% formamide. After 20 h at 42°C, the filter was washed twice with 3× SSC–0.1% sodium dodecyl sulfate at 37°C for 30 min and then twice with 0.1× SSC–0.1% sodium dodecyl sulfate at 37°C for 30 min and then autoradiographed at −80°C.

Conditions for in vitro reaction. FM3A nuclear extract was prepared as described previously (2). A 1-liter culture of FM3A cells at density of 4×10^5 to 5×10^5/ml was washed with phosphate-buffered saline. The cells were washed again with 20 ml of hypotonic buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5), 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.2 M sucrose and then suspended with 5 ml of the same buffer without sucrose and let stand at 0°C for 10 min. The nuclei were prepared by 40 to 50 strokes of Dounce homogenization followed by centrifugation at 3,000 × *g* for 5 min,

* Corresponding author.

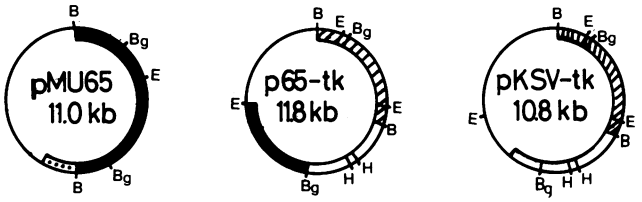

FIG. 1. Restriction map of plasmids used in this study. Abbreviations and symbols: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Bg, *Bgl*II; ▭, yeast *URA3* gene; ——, pBR322 (for pMU65) or pKB111 (for p65-tk); ■, mouse DNA; □, SV40 DNA; ▨, *tk* gene.

suspended with 2.5 ml of 50 mM HEPES buffer (pH 7.5) containing 10% sucrose, and immediately stored in liquid nitrogen. The nuclei were thawed and extracted with 100 mM NaCl at 0°C for 5 min. The nuclear extract was freed of insoluble material by centrifugation at 20,000 × *g* for 20 min. The reaction mixture (100 μl) contained 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.05 mM dATP, 0.05 mM dGTP, 0.05 mM dTTP, 1.5 μM [α-^{32}P]dCTP (410 Ci/mmol; 5,000 to 15,000 cpm/pmol), 3.75 mM ATP, 20 μl of FM3A nuclear extract (protein concentration, 2.5 mg/ml), and 0.2 μg of form 1 p65-tk. After incubation of the reaction mixture for 60 min at 37°C and subsequent digestion with proteinase K (10 μg) and sodium dodecyl sulfate (0.2%) for 20 min at 37°C, the DNA was precipitated with ethanol. The DNA was dissolved in 50 μl of 40 mM Tris–1 mM EDTA–5 mM sodium acetate (pH 7.8) and electrophoresed on 1% agarose gel in the same buffer. The gel was dried and autoradiographed on Fuji RXO-H X-ray film.

RESULTS

Replication of cloned mouse DNA in mouse cells. We have already cloned the mouse DNA fragment which can replicate in a SV40 T antigen-dependent system (5). The *Eco*RI-*Bgl*II region of this clone (pMU65) had sequences homologous to 13 nucleotides corresponding to the origin region of SV40 DNA replication. The *Eco*RI-*Bgl*II region of mouse DNA and the *tk* gene of herpes simplex virus were inserted into pKSV10 (Fig. 1). This clone, p65-tk, does not contain the origin of SV40 DNA replication. The SV40-derived sequences present in p65-tk are splicing and poly(A) addition signals. The three plasmids, p65-tk, pAGO that contains only the *tk* gene in pBR322, and p111-tk that contains the *tk* gene and the mouse DNA fragment inactive in a SV40 T antigen-dependent system (5), were respectively transfected into mouse FM3A^{tk-} cells by the liposome-mediated gene transfer technique, and cells were cultured in HAT medium. Two weeks after transfection, the FM3A^{tk+} transformants were isolated. Approximately 300 tk^+ transformants per 10^3 transfected cells were obtained. This novel transfection method will be reported elsewhere (Itani et al., submitted for publication). The low-molecular-weight DNA was extracted from the randomly selected FM3A^{tk+} cells that already had 60 population doublings and digested with *Dpn*I to eliminate the input p65-tk used for transfection. The p65-tk grown in a *dam*$^+$ *Escherichia coli* strain was methylated and thus rendered sensitive to cleavage by *Dpn*I. Since mammalian cells do not contain this methylase, DNA replication produces hemimethylated and unmethylated DNA, both of which are insensitive to cleavage by *Dpn*I. The digested DNA from the Hirt supernatant was analyzed by Southern blot hybridization with a ^{32}P-labeled p65-tk probe that contains the origin of pBR322 and β-lactamase gene common to all of the plasmids tested (Fig. 2). The autoradiogram showed form 1 and 2 of p65-tk in the transfected FM3A cells. The copy numbers were estimated as 100 to 200 copies per cell. On the other hand, p111-tk and pAGO gave no hybridizable bands (Fig. 2, lanes 7 and 8, respectively). These results clearly indicate that p65-tk was capable of replicating in mouse FM3A cells. The DNAs from 20 tk^+ transformants randomly chosen among approximately 300 transformants were checked to determine the existence of the p65-tk plasmid in the Hirt supernatant. Nineteen clones contained 100 to 500 copies of p65-tk per cell. Only one clone contained 20 to 30 copies of p65-tk (Fig. 2, lane 3). The next question was whether p65-tk was integrated into the chromosomal DNA in transformants. The high-molecular-weight DNA from transformants was isolated, digested with *Sal*I, transferred to a nitrocellulose filter after electrophoresis, and hybridized with the ^{32}P-labeled pBR322 probe that contains the origin and β-lactamase gene present in p65-tk. The results showed no hybridizable bands (data not shown), indicating that p65-tk was not integrated into the chromosomal DNA. The stability of p65-tk in the transformants was then examined. The DNA was isolated from cells after 60, 100, and 150 population doublings in culture with HAT medium and analyzed by Southern blot hybridization. p65-tk was stably present at 200 to 500 copies per cell in all of the clones tested. Without HAT selection, less than 1% loss of the plasmid per generation was observed. Next, to determine whether p65-tk was rearranged in the transformants, *E. coli* C600 was transformed with the DNA purified from Hirt supernatant of tk^+ transformant cells, and the plasmid DNA from the *E. coli* C600 was analyzed by agarose gel electrophoresis (Fig. 3). The results clearly show that rearrangement, if any, was under the detectable level. These results indicate that p65-tk can replicate in quite stable plasmid form during a long period, even without selective (HAT medium) conditions.

Replication of p65-tk in various cells. We used a liposome-mediated gene transfer technique that gives almost 100% transfection efficiency (Itani et al., submitted for publication) and is thus a quite efficient transient replication assay. For this assay, pARS65, which is p65-tk lacking the *tk* gene, was used. Besides FM3A cells, NS-1, a mouse myeloma cell line,

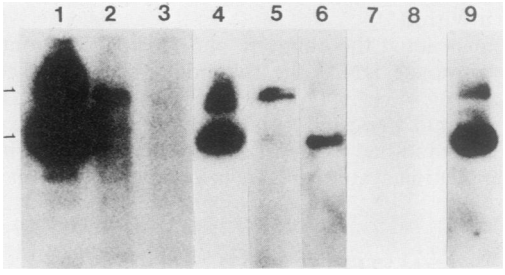

FIG. 2. Southern blot analysis of the DNA from Hirt supernatants in the transformants. The low-molecular-weight DNAs extracted from Hirt supernatants in the various clones of FM3A^{tk+} transformants after 60 population doublings were digested with *Dpn*I, electrophoresed on an 0.8% agarose gel, transferred to nitrocellulose filter by the method of Southern (21), and then hybridized with the nick-translated, ^{32}P-labeled *Eco*RI-*Bgl*II fragment of p65-tk. Lanes: 1, 10^4 copies of p65-tk per cell; 2 through 6, various FM3A^{tk+} clones; 7, FM3A^{tk+} clone transfected with p111-tk; 8, FM3A^{tk+} clone transfected with pAGO; 9, 5 × 10^2 copies of p65-tk per cell. The arrows indicate the positions of forms II and I of p65-tk from the top.

and HL-60, a human promyelocytic leukemia cell line, were transfected with pARS65. At 48 h after transfection, the DNA in the Hirt supernatant of the cells were analyzed by Southern blot hybridization after the DNA was digested with *Dpn*I and *Eco*RI to eliminate the input DNA and to linialize the replicated DNA (Fig. 4). The results clearly showed that pARS65 replicated in mouse and human cells. The copy numbers of pARS65 were approximately 200, 500, and 10,000 copies per cell in FM3A, NS-1, and HL-60 cells, respectively. These results suggest that origin sequences must be conserved at least between mouse and human DNAs.

Establishment of in vitro DNA replication system. To analyze the molecular mechanism of DNA replication, in vitro systems are quite powerful as demonstrated in procaryotes (18) and in eucaryotic viral systems including adenovirus (8) and SV40 (3, 4, 19). Therefore, we tried to establish an in vitro DNA replication system to analyze p65-tk DNA replication in mouse cells. The extract was made from FM3A cells in the same manner as that in the SV40 DNA replication system we established previously. The system was consisted of nuclear extract, four deoxynucleotide triphosphates, ATP, $MgCl_2$, and exogeneously added form I p65-tk. The important point is that this system is not dependent upon SV40 T antigen. After reaction at 37°C for 60 min, the products were visualized by neutral agarose gel electrophoresis followed by autoradiography (Fig. 5). p65-tk was a good template and gave rise to two major bands, form I and form II, and replicative intermediates above form II (Fig. 5, lane 1). On the other hand, the reactions on pKSV-tk, which is p65-tk lacking the *Bgl*II-*Eco*RI mouse DNA fragment and without plasmid DNA, yielded no detectable bands on the gel (Fig. 5, lanes 2 and 3, respectively). When a time course experiment was carried out, the typical replication patterns of double-stranded circular DNA were observed as shown previously in the SV40 DNA replication system (3). One round of replication required about 20 min, which is quite similar to that observed in vivo. After 10 min, the replicative intermediates accumulated. After 60 min, the products of the in vitro reaction were mainly forms I and II (Fig. 5). Approximately 150 pmol of [^{32}P]dCMP per μg of the added p65-tk was incorporated in 1 h of incubation, indicating that about 10% of the added DNA was used as a template. These data clearly show that p65-tk replicates in vitro in mouse FM3A nuclear extract. This conclusion was further supported in the experiments described below.

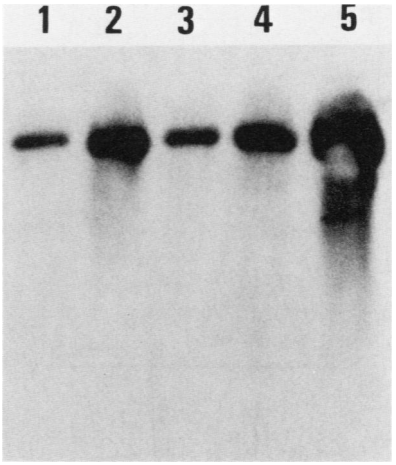

FIG. 4. Southern blot analysis of the DNA from Hirt supernatants of various transfected cells. Samples of 10^6 FM3A, NS-1, and HL-60 cells were transfected with 5 μg of pARS65. At 48 h after transfection, the low-molecular-weight DNA was extracted from Hirt supernatants, digested with *Dpn*I and *Eco*RI, electrophoresed on 1% agarose gel, transferred to nitrocellulose filters by the method of Southern (21), and then hybridized with ^{32}P-labeled p65-tk probe. Lanes: 1, 10^2 copies per cell; 2, 10^3 copies per cell; 3, FM3A; 4, NS-1; 5, HL-60.

Evidence for semiconservative replication. To determine whether in vitro products were produced by semiconservative replication or repair synthesis, the DNA was synthesized in a reaction mixture containing bromodeoxy UTP in place of dTTP and analyzed by alkaline and neutral CsCl

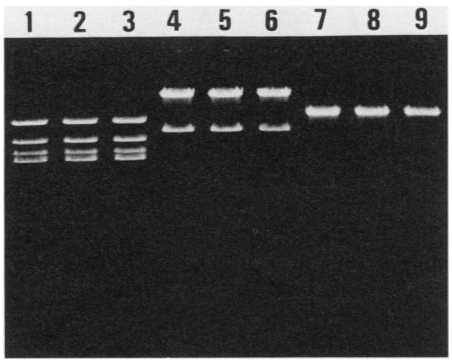

FIG. 3. Ethidium bromide staining patterns of DNA derived from Hirt supernatant of tk^+ transformants. The low-molecular-weight DNA was extracted from the Hirt supernatants of two FM3A^{tk+} transformants. *E. coli* C600 was transformed to the ampicillin-resistant phenotype with the above DNAs. The plasmid DNAs were extracted from *E. coli* transformants, analyzed by agarose gel electrophoresis, and visualized under UV light. Lanes: 1, 4, and 7, p65-tk; 2, 5, and 8, DNA from clone 1; 3, 6, and 9, DNA from clone 2; 1, 2, and 3, digested with *Eco*RI and *Bgl*II; 4, 5, and 6, digested with *Bam*HI; 7, 8, and 9, digested with *Pst*I.

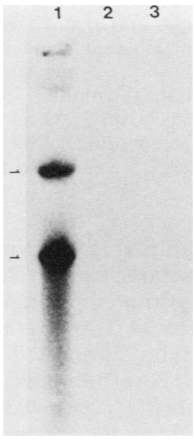

FIG. 5. DNA synthesis in vitro on p65-tk independent of SV40 T antigen. Synthesis independent of SV40 T antigen was carried out with 0.2 μg of each DNA as a template. After 60 min at 37°C, the reaction mixture was treated with proteinase K and sodium dodecyl sulfate, and the DNA was precipitated with ethanol after phenol extraction. DNA was visualized by autoradiography after agarose gel electrophoresis. Reactions were performed with p65-tk (lane 1), pKSV-tk (lane 2), and without DNA (lane 3) as a template. The horizontal bars represent the positions of form II and I DNAs from the top.

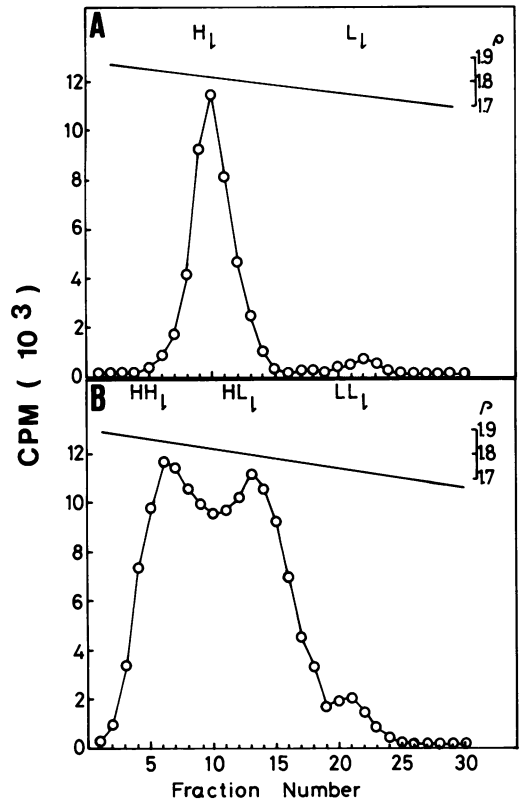

FIG. 6. Isopycnic centrifugation of DNA synthesis in vitro in the presence of bromodeoxy UTP. A standard reaction mixture containing 30 μM each dATP, dGTP, and bromodeoxy UTP (in place of dTTP) and 1.5 μM [α-^{32}P]dCTP was carried out on p65-tk for 60 min at 37°C. The DNA was extracted with phenol after treatment of the mixture with proteinase K (500 μg/ml) for 20 min at 37°C and precipitated with ethanol. The density of the DNA sample containing 25 mM Tris (pH 7.4), 1 mM EDTA, and 0.1 M NaCl was adjusted to 1.8 g/ml by the addition of CsCl and centrifuged in an VTi65 rotor at 47,000 rpm for 20 h at 25°C. A, Centrifugation under alkaline conditions (150 mM K_3PO_4 [pH 12.5], 1.5 mM EDTA): B, centrifugation under neutral conditions (20 mM Tris hydrochloride [pH 7.5], 1 mM EDTA). After fractionation, the acid-insoluble radioactivity was counted, and the density was measured. Arrows indicate the expected positions for fully substituted, hybrid, and unsubstituted DNAs (left to right, respectively). The total incorporated dCMP amounts estimated by the counting 0.1 ml of the reaction were 140 and 160 pmol/μg of added DNA in A and B, respectively.

equilibrium centrifugation (Fig. 6). When p65-tk was used as a template in the reaction, almost all the in vitro product banded at the fully substituted position under alkaline denaturing conditions (Fig. 6A). Approximately 40% of the in vitro products were fully substituted by bromouracil, 10% were intermediate between fully and half substituted, 45% banded at a hybrid density which was half substituted, and only 5% had unsubstituted density under neutral undenaturing conditions (Fig. 6B). This result clearly indicates that approximately 95% of the in vitro products in the reaction with p65-tk as a template were derived from semiconservative DNA replication.

Determination of the initiation point in the reaction. p65-tk consists of fragments from mouse, herpes simplex virus, and bacterial plasmid DNAs. To determine the initiation point in the reaction on p65-tk, newly synthesized DNA labeled for 5, 10, and 60 min was extracted and digested with *Eco*RI, *Bgl*II, *Bam*HI, and *Hin*dIII sequentially, and the distribution of radioactivity on each fragment was examined by agarose gel electrophoresis (Fig. 7). Digestion of p65-tk with these combinations of enzymes yielded mainly three large fragments. The ratio of the radioactivity of each fragment was normalized to the ratio of the nucleotide numbers of each fragment. Therefore, the value 1 means that all sequences are equally labeled. In the first 5 min of the reaction, a very high ratio was obtained on 2.5 kb of the *Eco*RI-*Bgl*II fragment (ratio, 7.78). After 60 min of the reaction, the ratio of each fragment was almost 1, indicating that the label became more evenly incorporated on each fragment of p65-tk. These results show that the initiation of DNA replication of the plasmid carried out in the mouse nuclear extract started from the mouse DNA fragment in it and that the DNA replication was bidirectional.

Existence of sequences similar to p65-tk all over the mouse genomic DNA. The total DNA was extracted from FM3A cells and digested with *Eco*RI or *Bam*HI. Then Southern blot hybridization was carried out with the ^{32}P-labeled *Eco*RI-*Bgl*II fragment of p65-tk as a probe (Fig. 8). The autoradiogram shows the hybridizable smear ranging from small- to large-molecular-weight fragments. To eliminate the possibility that the 2.5-kb *Eco*RI-*Bgl*II fragment contains repeated sequences such as the *Alu* and *Kpn*I family of human DNA or the B1 and B2 family of mouse DNA that should give the hybridizable smear after Southern blot hybridization, BLUR8 (*Alu* family), pE3 (*Kpn*I family A, B, C regions), pE5 (*Kpn*I family D region), pSV2β (B1 family), and pBII (B2 family) were digested with restriction enzymes to separate repeated DNA fragments and pBR322 and analyzed by Southern blot hybridization with the ^{32}P-labeled *Eco*RI-*Bgl*II fragment of p65-tk. The hybridizable bands were undetectable in all cases. These results show that the sequences similar to the *Eco*RI-*Bgl*II mouse DNA fragment of p65-tk,

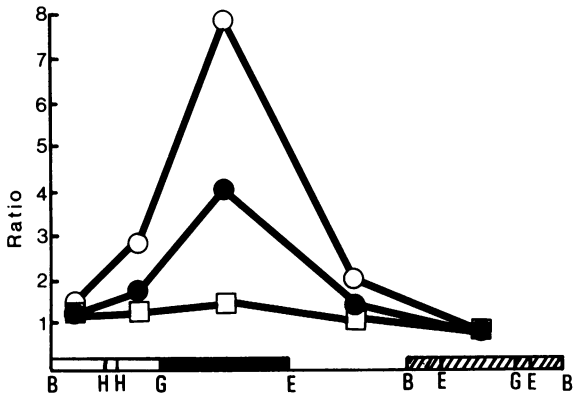

FIG. 7. Kinetics of labeling of various regions of p65-tk. The in vitro reaction was carried out with 0.2 μg of p65-tk. After incubation of the reaction mixture for 5, 10, or 60 min at 37°C and subsequent digestion with proteinase K and sodium dodecyl sulfate for 20 min at 37°C, the DNAs were dissolved in water, mixed with 1 μg of cold p65-tk and digested sequentially with *Eco*RI, *Bgl*II, *Bam*HI, and *Hin*dIII. DNA fragments were then separated on 1% agarose gel containing 0.5 μg of ethidium bromide per ml and cut out after illumination under UV light, and the radioactivity was counted. The ratio of incorporated radioactivity is expressed as a function of the position of each fragment on DNA. The ratio of the *tk* gene fragment was arbitrarily set as 1, and the other values were normalized to it. Symbols: ○, 5-min reaction; ●, 10-min reaction; □, 60-min reaction. A map of p65-tk is shown at the bottom. Symbols: ▭, SV40 DNA; ■, mouse DNA; ——, pKB111; ▨, *tk* gene.

which has autonomous replicating capacity in mouse cells, were present in almost all of the genomic DNA of mouse cells.

DISCUSSION

Several investigators have tried to search for ARSs from various organisms. ARS elements have been isolated from *S. cerevisiae*, *Neurospora crassa*, *Tetrahymena thermophila*, and *Xenopus laevis* (7, 9, 13–15, 17, 20, 22–26). The frequency of ARS elements isolated from the yeast genome resembles the average frequency of replication initiation observed in chromosomes (9). The simplest interpretation of their function appears to be that they provide replication initiation sites for plasmids that lack them; hence, they may be chromosomal origins of replication. Therefore, isolation of ARS elements from much higher organisms would be necessary for understanding the mechanism of initiation of DNA replication.

We have already cloned a mouse DNA fragment that could replicate in a SV40 T antigen-dependent system (5). The *Eco*RI-*Bgl*II mouse DNA fragment of this clone, pMU65, had sequences homologous to the origin containing the region of SV40 DNA replication. This cloned fragment coupled with the *tk* gene (p65-tk) was introduced into mouse FM3A^{tk-} cells. The FM3A^{tk+} cells harbored p65-tk at 100 to 200 copies per cell and were resistant to digestion with *Dpn*I. This indicates that p65-tk could replicate autonomously in mouse cells after transfection. The question raised was whether p65-tk replicates during the S phase or throughout the cell cycle. When cells were harvested at the logarithmic, growing phase, the replicated p65-tk or pARS65 plasmids were easily detected at high copy numbers. On the other hand, when cells were harvested at the stationary, nonproliferating phase, low copy numbers of p65-tk and pARS65 were detected. This suggested that replication of p65-tk occurs only during the S phase. However, more critical experiments should be done to confirm this possibility. The replication activity of p65-tk was further supported by the in vitro DNA replication experiments. The p65-tk could replicate semiconservatively, and the initiation of DNA replication started from the mouse DNA fragment in the reaction performed by the mouse FM3A nuclear extract. These results suggest that cloned mouse fragment must possess the origin region of mouse DNA replication itself.

It is generally known that initiation sites of DNA replication in mammalian cells are distributed throughout the genome at average intervals of 10 to 20 kb (18). Therefore, it is reasonable that the sequences similar to p65-tk were present in the mouse FM3A DNA throughout the genome if the sequence focused on here is indeed one of the sequences necessary for initiation of DNA replication. pMU65 containing the *Eco*RI-*Bgl*II region of p65-tk also replicated in monkey Cos cells, which constitutively produce the SV40 T antigen that is essential to replication of SV40 origin-containing plasmids (5). Therefore, it would be important to determine whether the same sequences are necessary for the initiation of DNA replication. Jelinek et al. have made it apparent from nucleic acid sequence studies that there are nucleotide similarities between the *Alu* family by interspersed repeated sequences that are present approximately 300,000 times throughout the eucaryotic genome and the origin region of SV40, BK virus, and polyomavirus, suggesting that the *Alu* family might function as the origin of DNA replication in mammalian cells (16). Therefore, we would be able to make a possibility that the same or similar sequences present in p65-tk used here may function as ARSs in mouse DNA replication as well as SV40 DNA replication. Sequencing studies and the mutational analysis of the *Eco*RI-*Bgl*II region of p65-tk should be done.

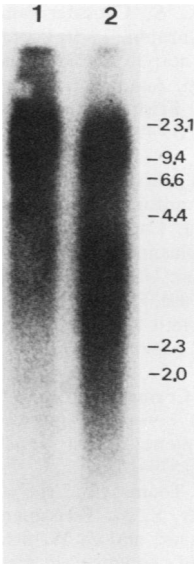

FIG. 8. Southern blot analysis of the FM3A total DNA. Total DNA extract from FM3A^{tk-} cells was digested with *Eco*RI (lane 1) or *Bam*HI (lane 2), separated on an 0.8% agarose gel, transferred to the nitrocellulose filter, and hybridized with the nick-translated ^{32}P-*Eco*RI-*Bgl*II fragment of p65-tk. The conditions of hybridization are described in Materials and Methods.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Education, Science, and Culture in Japan.

We thank N. Yamaguchi for the critical reading of the manuscript.

LITERATURE CITED

1. **Aizawa, D., H. Koyama, and T. Seno.** 1981. Resistance to methotrexate in thymidylate synthetase-deficient mutants of cultured mouse mammary tumor FM3A cells. Cancer Res. **41**:1497–1501.
2. **Ariga, H.** 1984. Replication of cloned DNA containing the *Alu* family sequence during cell extract-promoting simian virus 40 DNA synthesis. Mol. Cell. Biol. **4**:1476–1482.
3. **Ariga, H.** 1984. Identification of the replicative intermediates of SV40 DNA replication in vitro. Nucleic Acids Res. **12**: 6053–6062.
4. **Ariga, H., and S. Sugano.** 1983. Initiation of simian virus 40 DNA replication in vitro. J. Virol. **48**:481–491.
5. **Ariga, H., Z. Tsuchihashi, M. Naruto, and M. Yamada.** 1985. Cloned mouse DNA fragments can replicate in a simian virus 40 T antigen-dependent system in vivo and in vitro. Mol. Cell. Biol. **5**:563–568.
6. **Beggs, J. D.** 1978. Transformation of yeast by a replicating hybrid plasmid. Nature (London) **275**:104–109.
7. **Broach, J. R., and J. B. Hicks.** 1980. Replication and recombination functions associated with the yeast plasmid, 2u circle. Cell **21**:501–508.
8. **Challberg, M. D., and T. J. Kelly, Jr.** 1979. Initiation of adenovirus DNA replication in vitro. Proc. Natl. Acad. Sci. USA **76**:655–659.
9. **Chan, C. S., and B. Tye.** 1980. Autonomous replicating sequences in *Saccaromyces cerevisiae*. Proc. Natl. Acad. Sci. USA **11**:6329–6333.

10. **Colbere-Garapin, F., S. Chousterman, F. Horodniceanu, F. Kourilsky, and A. Garapin.** 1979. Cloning of the active thymidine kinase gene of herpes simplex virus type 1 in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **76**:3755–3759.
11. **Gluzman, Y., R. J. Frisque, and J. Sambrook.** 1980. Origin-defective mutant of SV40. Cold Spring Harbor Symp. Quant. Biol. **44**:293–300.
12. **Hirt, J. B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
13. **Hsiano, C.-L., and J. Carbon.** 1979. High frequency transformation of yeast by plasmids containing the cloned *ARG4* gene. Proc. Natl. Acad. Sci. USA **76**:3829–3833.
14. **Hughes, K., M. E. Case, R. Geever, D. Vapnek, and N. H. Giles.** 1983. Chimeric plasmid that replicates autonomously in both *Escherichia coli* and *neurospora crassa*. Proc. Natl. Acad. Sci. USA **80**:1053–1057.
15. **Hyman, B. C., J. H. Cramer, and R. H. Rownd.** 1982. Properties of a *Saccaromyces cerevisiae* mitochondrial DNA segment conferring high-frequency yeast transformation. Proc. Natl. Acad. Sci. USA **79**:1578–1582.
16. **Jelinek, W. R., T. P. Toomey, L. Leinward, C. H. Duncan, P. A. Biro, P. V. Chouday, S. M. Weissman, C. M. Rubin, C. M. Houck, P. L. Deininger, and C. W. Schmid.** 1980. Ubiquitous, interspersed repeated sequences in mammalian genome. Proc. Natl. Acad. Sci. USA **77**:1398–1402.
17. **Kiss, G. B., A. A. Amin, and R. E. Pearlman.** 1981. Two separate regions of the extrachromosomal ribosomal deoxyribonucleic acid of *Tetrahymena thermophila* enable autonomous replication of plasmids in *Saccaromyces cerevisiae*. Mol. Cell. Biol. **1**:535–543.
18. **Kornberg, A.** 1980. DNA replication. W. H. Freeman & Co., San Francisco.
19. **Li, J. J., and T. J. Kelly, Jr.** 1984. Simian virus 40 DNA replication in vitro. Proc. Natl. Acad. Sci. USA **81**:6973–6977.
20. **Rochaix, J.-D., J. van Dillewijn, and M. Rahire.** 1984. Construction and characterization of autonomously replicating plasmids in the green unicellular alga *Chlamydomonas reinhardii*. Cell **36**:925–931.
21. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **93**: 503–517.
22. **Stinchcomb, D. T., M. Thomas, I. Kelly, E. Selker, and R. W. Davis.** 1980. Eukaryotic DNA segments capable of autonomous replication in yeast. Proc. Natl. Acad. Sci. USA **77**:4559–4563.
23. **Stohl, L. L., and A. M. Lambowitz.** 1983. Construction of a shuttle vector for a filamentous fungus *Neurospora crassa*. Proc. Natl. Acad. Sci. USA **80**:1058–1062.
24. **Struhl, K., D. T. Stinchcomb, S. Scherer, and R. W. Davis.** 1979. High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA **76**:1035–1039.
25. **Zakian, V. A.** 1981. Origin of replication from *Xenopus laevis* mitochondrial DNA promotes high-frequency transformation of yeast. Proc. Natl. Acad. Sci. USA **78**:3128–3132.
26. **Zakian, V. A., and J. F. Scott.** 1982. Construction, replication, and chromatin structure of TRP1 R1 circle, a multiple-copy synthetic plasmid derived from *Saccaromyces cerevisiae* chromosomal DNA. Mol. Cell. Biol. **2**:221–232.